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Polyethylenimine-cyclodextrin-tegafur conjugate shows anti-cancer activity and a potential for gene delivery*

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Abstract: Polyethylenimine-cyclodextrin-tegafur (PEI-CyD-tegafur) conjugate was synthesized as a novel multi-functional prodrug of tegafur for co-delivery of chemotherapeutic agent tegafur and enhanced green fluorescent protein (EGFP) reporter plasmid DNA. Conjugation of tegafur to PEI-CyD via chemical linkage was characterized by ^{1}H NMR spectrometry and ultraviolet (UV) spectrometry. PEI-CyD-tegafur was able to condense plasmid DNA into complexes of around 150 nm with positive charge at the N/P ratio of 25, in accordance with electron microscopy observation of compact and monodisperse nanoparticles. The results of in vitro experiments showed enhanced cytotoxicity and considerable transfection efficiency in B16F10 cell line. Therefore, PEI-CyD-tegafur may have great potential as a co-delivery system with anti-cancer activity and potential for gene delivery.

Key words: Polyethylenimine, β-Cyclodextrin, Tegafur, Co-delivery, Gene therapy
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1 Introduction

Tegafur, an orally active prodrug of clinical anti-cancer drug 5-fluorouracil (5-FU) (Malet-Martino and Martino, 2002), was developed for cancer treatment with improved bioavailability and less drug resistance in cancer cells. Tegafur can be gradually converted to 5-FU in vivo by the cytochrome P450 enzyme system in the liver, by thymidine phosphorylase in tumor tissue, and by spontaneous degradation (Cao *et al.*, 1995), and can maintain the concentration of 5-FU in blood over a prolonged period (Friedman and Ignoffo, 1980). It is also widely used as a component in Uftoral (UFT) (Akahoshi *et al.*, 1998) and S-1 (Takechi *et al.*, 1997); however, those chemotherapeutic agents are suggested to have severe adverse effects, especially systematic cytotoxicity. Due to the cytotoxicity of tegafur, clinical studies show that administration of S-1 increases the incidence of neutropenia (Yamanaka *et al.*, 2007a; 2007b; 2008) and pneumonia (Shitara *et al.*, 2007; Tada *et al.*, 2007), while administration of UFT induces anorexia, nausea, diarrhoea, epigastric pain (Akay *et al.*, 2004), stomatitis, skin pigmentation (Seishima *et al.*, 2000), myelosuppression, and dizziness (Ota *et al.*, 1988).

Nanoparticle drug carriers and other novel prodrugs were developed to reduce systematic toxicity. Polymeric drug delivery systems (Arias *et al.*, 2007; 2008a; 2008b; Zeng *et al.*, 2009) and chemically-synthesized derivatives (Stokes *et al.*, 2002; Zhang *et al.*, 2007; Engel *et al.*, 2008) can perform controlled release of tegafur upon exposure to specific

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conditions and thus improve anti-cancer activity. Compared to those prodrugs mentioned above, polyethylenimine-cyclodextrin (PEI-CyD) copolymer has not only the ability to deliver drugs (Zhao *et al.*, 2009) as well as plasmid DNA (Tang *et al.*, 2006) but also the potential to perform co-delivery of genes and anti-cancer drugs. With this advantage, the combination of chemotherapy and gene therapy was likely to be conducted by multiple functional components in a single conjugate compound. We aim to create a delivery system that could: (1) be easy to synthesize in a few steps; (2) be dissolved in water; (3) inhibit cancer cells with a hydrolyzed drug; (4) condense plasmid DNA (pDNA) to form complex within a reasonable size; (5) deliver the gene plasmid DNA into cancer cells; (6) protect the gene from lysosomal enzymolysis.

In order to make those features available, PEI-CyD-tegafur conjugate was synthesized as a novel multifunctional prodrug of tegafur. Evaluation of anti-cancer activity and gene delivery potential of PEI-CyD-tegafur conjugate and its drug/gene co-delivery ability is carried out in this study.

2 Materials and methods

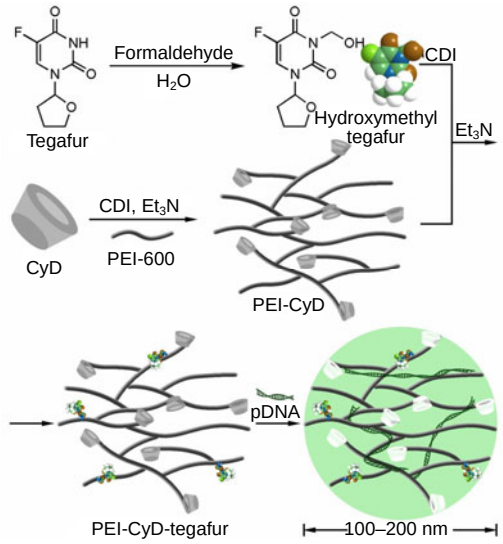
2.1 Materials

Polyethylenimine (PEI, molecular weight 600 Da and 25 kDa), β-cyclodextrin (CyD), triethylamine (Et_3N), dimethyl sulfoxide (DMSO), *N*,*N*'-carbonyldiimidazole (CDI), and formaldehyde were purchased from Sigma-Aldrich. Ethyl acetate (EtOAc) and hexane were purchased from Hushi Chemical Corporation (Shanghai, China). Plasmid DNA pGL-3 was purchased from Promega Corporation (Madison, WI). COS7, B16F10, and HT29 cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD) and were cultured in complete medium containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) streptomycin/penicillin as described by ATCC.

2.2 Synthesis of conjugate

2.2.1 Synthesis of hydroxymethyl tegafur

Hydroxymethyl tegafur was synthesized to provide a hydroxyl group for tegafur to conjugate with PEI-CyD (Fig. 1). A solution of tegafur (1.00 g, 5.00 mmol) and 35% formaldehyde (0.50 ml, 5.80 mmol, 1.16 equiv) was stirred for 1 h in a preheated oil bath at 50 °C. The mixture was then evaporated, and the oily colorless residue was dissolved in EtOAc and purified through silica gel column eluated with EtOAc/hexane 3:1 (v/v). The eluent was evaporated almost to dryness and, upon addition of diethyl ether, the product was crystallized as white crystals.



CDI: *N*,*N*'-carbonyldiimidazole; CyD: β-cyclodextrin; Et_3N: triethylamine; PEI-600: polyethylenimine (molecular weight 600 Da)

Fig. 1 Synthesis of PEI-CyD-tegafur conjugate

2.2.2 Synthesis of PEI-CyD copolymer

PEI-CyD copolymer was synthesized as described previously in (Tang *et al.*, 2006) (Fig. 1). CyD (2.17 g, 1.91 mmol) and CDI (2.43 g, 15.0 mmol) were dissolved in 20 ml of DMSO, and then mixed with 200 μl of Et_3N. The mixture was stirred at room temperature for 3 h under nitrogen. PEI-600 (8.83 g, 14.7 mmol) was dissolved in 15 ml of DMSO. After addition of 200 μl of Et_3N, the PEI solution was added dropwise to CyD-CDI over 2.5 h with stirring, followed by an overnight reaction. The crude product was dialyzed in water for two days and freeze-dried for another two days.

2.2.3 Synthesis of PEI-CyD-tegafur conjugate

The PEI-CyD-tegafur conjugate was synthesized as shown in Fig. 1. Hydroxymethyl tegafur (64.2 mg, 0.28 mmol) and CDI (69.7 mg, 0.42 mmol, 1.50 equiv) were dissolved in 2.50 ml of DMSO, mixed with

200 μl of Et_3N, and then stirred for 3 h under nitrogen at room temperature. PEI-CyD (85.2 mg) dissolved in DMSO was slowly added to tegafur-CDI, and after adding 200 μl of Et_3N, the mixture was further stirred overnight under nitrogen. Then the crude product was dialyzed in water for one day and freeze-dried.

2.3 Characterization of PEI-CyD-tegafur conjugate

2.3.1 ^{1}H NMR analysis

The structures of PEI-CyD and PEI-CyD-tegafur were characterized by ^{1}H NMR. The ratio of CyD and PEI in the polymer sample was determined from ^{1}H NMR spectra using integral values obtained for the -H protons of the CyD rings and $-CH_2CH_2NH-$ protons of PEI. The drug loading condition of PEI-CyD-tegafur was analyzed by comparing ^{1}H NMR spectra of tegafur, PEI-CyD and PEI-CyD-tegafur. The ^{1}H NMR analysis was carried out with 10 mg of sample dissolved in 0.5 ml of deuterium DMSO (DMSO-d_6) or deuterium oxide (D_2O) in a Bruker 400 MHz NMR spectrometer with eight scans at room temperature.

2.3.2 Ultraviolet (UV) spectroscopy

UV spectroscopy was conducted to determine the conjugation property and drug loading amount of PEI-CyD-tegafur. The spectra of tegafur, PEI-CyD and PEI-CyD-tegafur were recorded between 230 and 300 nm using a UV-Vis spectrophotometer at room temperature. A calibration curve was established by measuring the absorbance of standard tegafur solutions with different concentrations at 272 nm. Drug loading amount was calculated by determining absorbance of PEI-CyD-tegafur at 274 nm.

2.4 Characterization of PEI-CyD-tegafur/DNA complex

2.4.1 DNA retardation assay

To obtain series of polymer/DNA complexes with different N/P ratios, 10 μl of plasmid DNA solution (containing 1 μg of pGL-3 pDNA in filtered distilled water) and 10 μl of polymer solution (containing varying amounts of polymer in filtered distilled water) were mixed. Complexes were allowed to form for 30 min at room temperature and the resulting solutions, mixed with loading buffer, were loaded onto 1% agarose gel for gel electrophoresis conducted in 1× TAE buffer (40 mmol/L Tris acetate and 1 mmol/L EDTA) at 90 V for 45 min in a Sub-Cell system (Biorad Laboratories, CA). DNA bands were visualized by a UV transilluminator and were recorded by GeneSnap imaging system (Syngene, Cambridge, UK).

2.4.2 Complex size and zeta-potential measurement

Particle size and zeta-potential measurement was performed on a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA) with a laser light wavelength of 633 nm at a scattering angle of 173°. DNA-polymer complexes with different N/P ratios were formed in 0.5 ml Eppendorf tubes by adding 10 μl of plasmid DNA solution (containing 1 μg pGL-3 pDNA in filtered distilled water) to 10 μl of polymer solution (containing varying amounts of polymer in filtered distilled water). Complexes were allowed to form for 30 min at room temperature, and were then diluted to 1 ml with filtered distilled water. The particle size was measured at 25 °C in a glass cuvette. For zeta-potential measurement, the polymer/DNA complexes were formed in a 0.1 mol/L KCl solution and the measurements were performed using a capillary zeta-potential cell in automatic mode.

2.4.3 Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies

To obtain the optical and structural properties of the polymer/DNA complexes, TEM and SEM were used to visualize the complexes. The complexes were observed using a Tecnai 10 TEM (Philips Electron Optics, Eindhoven, NL) and photographed with a Gatan Erlangshen 500 W digital camera (Pleasanton, CA). For SEM visualization, the gold-coated complexes were observed using a Cambridge Stereoscan 260 SEM (Cambridge, UK) at 20 kV.

2.5 Evaluation of in vitro cytotoxicity

In vitro cytotoxicities of tegafur and PEI-CyD-tegafur conjugate on HT29, B16F10, and COS7 cell lines were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into 96-well microtiter plates (Costar, Corning Co., NY) at the density of 8×10^3 cells in 200 μl complete medium per well. After incubation for 20 h, the cultural medium was replaced by complete medium with different concentrations of

selected chemicals. After further incubation for 72 h, 100 μl of 10% sterilized MTT (5 mg/ml) stock solution in serum-free medium was added to each well. After 4 h, unreacted dye was removed by aspiration, and the formazan crystals were dissolved in 100 μl DMSO per well and measured spectrophotometrically in a microplate reader (Spectra Plus, TECAN) at the wavelength of 570 nm. The relative cell viability related to control cells cultured in medium without chemicals was calculated by

$$V=\frac{A_{\text{experimental}}-A_{\text{blank}}}{A_{\text{control}}-A_{\text{blank}}}\times 100\%, \quad (1)$$

where V is the cell viability (%), $A_{\text{experimental}}$ is the absorbance of the wells culturing cells treated with chemicals, A_{blank} is the absorbance of media blanks, and A_{control} is the absorbance of the wells culturing cells treated with chemical-free 10% FBS-medium. The 50% inhibitory concentration (IC_{50}) was determined by fitting data to the following equation:

$$V=\frac{100}{1+(C_{\text{tegafur}}/\text{IC}_{50})^{p}}, \quad (2)$$

where C_{tegafur} is well concentration (μg/ml) of tegafur or tegafur equivalent of PEI-CyD-tegafur conjugate, and p defines the slope of the sigmoid curve. Nonlinear curve-fitting was performed using Origin version 7.5 (OriginLab, MA) and the results presented were the average of independent experiments.

2.6 Evaluation of in vitro transfection

For in vitro transfection studies, B16F10 and COS7 cell lines were used to evaluate the EGFP expression. The cells were seeded at a density of 3×10^4 cells in 500 μl complete medium per well into a 24-well plate within 24 h. Plasmid DNA solution (10 μl; containing 1 μg plasmid pEGFP-N1) in phosphate-buffered saline (PBS) and 10 μl of polymer solution in PBS were mixed to obtain the polymer/DNA complexes with the N/P ratio of 25. After 30 min, the complex solutions were diluted to 500 μl with serum-free medium to obtain transfection medium. Transfection was performed as the complete medium was replaced with the transfection medium. After 4 h of incubation, the transfection medium was removed and then the cells were incubated in serum-containing medium for another 48 h. In the end, the cells were washed with warm PBS solution twice and imaged under an inverted fluorescence microscope (Eclipse Ti, Nikon Corp., Japan) excited with a 488-nm blue laser.

3 Results

3.1 Synthesis and characterization of PEI-CyD-tegafur conjugate

Hydroxymethyl tegafur, PEI-CyD copolymer, and PEI-CyD-tegafur were synthesized as shown in Fig. 1. CyD was activated by CDI and CyD-CDI was conjugated to PEI to obtain PEI-CyD copolymer via polycondensation reaction. The hydroxyl groups of CyD were coupled to the amine groups of PEI resulting in a one-carbon spacer to form the PEI-CyD polymer. Tegafur was converted to hydroxymethyl tegafur to provide an active hydroxyl group which could be activated by CDI. Tegafur-CDI compound was then conjugated to PEI-CyD copolymer via amido linkage. The amido linkage was formed by carbonyl group on CDI and primary amine on PEI.

^{1}H NMR spectrometry in D_2O was used to analyze the molar ratio of PEI-600/CyD (Fig. 2b) as previously described. The peaks at δ 2.3–2.8 were assigned to protons of -CH_2-CH_2-NH- from PEI-600 and the peak at δ 4.8 was assigned to C1 hydrogen of CyD. Stoichiometry calculated of integral values of PEI-600 (-CH_2-CH_2-NH-) and the C1 hydrogen of CyD (1:8.52) suggested the molar ratio of 1:1 between PEI and CyD. ^{1}H NMR spectrometry in DMSO-d_6 was used to confirm products' identities. The characteristic peaks at δ 7.86 and 5.90 were observed in the spectrum of tegafur (Fig. 2a). The spectrum of PEI-CyD-tegafur (Fig. 2c) shows the peaks at δ 7.78 and 5.85 attributed to tegafur, which indicates that tegafur was successfully conjugated to CyD-PEI copolymer.

The drug loading amount of the conjugate was calculated from the UV absorbance value of PEI-CyD-tegafur solution and the calibration standards of tegafur solutions. The calibration curve was plotted to determine the relation of absorbance and concentration, and the equation by linear fit was produced as shown below:

$$A_{\text{tegafur}}=0.07937\cdot C_{\text{tegafur}}+0.02322, \quad R^2=0.9969,$$

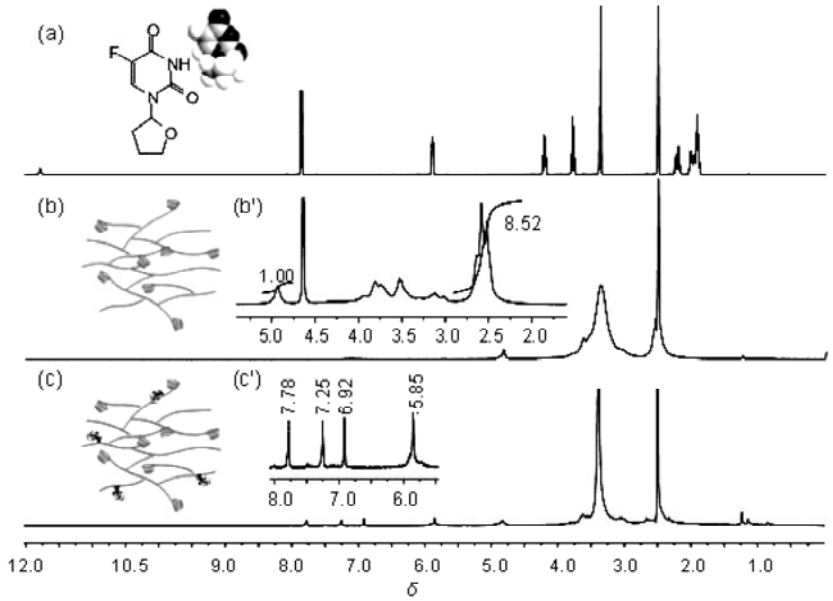


Fig. 2 ^{1}H NMR spectra of tegafur (a), PEI-CyD copolymer (b), and PEI-CyD-tegafur conjugate (c) in DMSO-d_6
(b′) shows the ^{1}H NMR spectrum of PEI-CyD copolymer in D_2O from which PEI-600/CyD molar ratio was calculated; (c′) shows the zoom spectrum of PEI-CyD-tegafur ranging from δ 5.6 to δ 8.0, which indicates the conjugation of tegafur to PEI-CyD copolymer

where $A_{tegafur}$ defines the absorbance of the tegafur solutions and $C_{tegafur}$ is the concentration of those solutions. Since PEI-CyD solution had little absorbance ranging from 245 to 300 nm, existence of PEI-CyD in PEI-CyD-tegafur conjugate would not affect the calculation of drug loading amount. From the calibration curve and the absorbance value of PEI-CyD-tegafur at 274.1 nm, tegafur loading amount of the conjugated was then calculated to be 0.51%. The UV spectra of tegafur and PEI-CyD-tegafur solutions were also the evidence of the conjugation of tegafur to PEI-CyD polymer. The absorbance peak of PEI-CyD-tegafur solution at 274.1 nm shows about 2 nm shift, compared to the peak of tegafur solution at 272.3 nm (Fig. 3). The red shift suggests the conjugation of tegafur to PEI-CyD by chemical linkage instead of simple embedding.

3.2 Characterization of PEI-CyD-tegafur/DNA complex

To evaluate the ability to form polymer/DNA complex, a DNA retardation assay was performed by agarose gel electrophoresis at various N/P ratios. PEI-CyD polymer was able to condense plasmid DNA into nanoparticle at the N/P ratio of 3 (Fig. 4a) and PEI-CyD-tegafur conjugate showed similar condensation ability as PEI-CyD (Fig. 4b). Although conjugation of tegafur to PEI-CyD slightly reduced DNA condensation capability, PEI-CyD-tegafur still had the ability to retard DNA completely at relatively low N/P ratios, indicating that density of primary amino groups on PEI-CyD is high enough to condense DNA, which will be only affected slightly by drug conjugation.

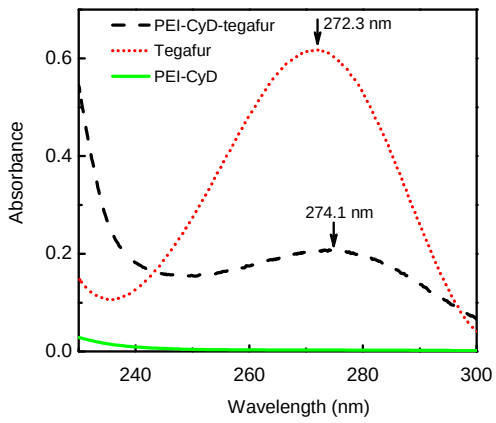


Fig. 3 UV spectra of PEI-CyD-tegafur conjugate, tegafur, and PEI-CyD copolymer solutions
The absorbance of PEI-CyD-tegafur and tegafur solutions peaks at 274.1 and 272.3 nm, respectively

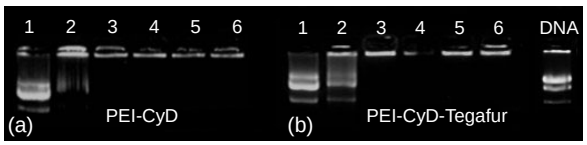


Fig. 4 DNA retardation assays of PEI-CyD (a) and PEI-CyD-tegafur (b) with increasing N/P ratios
PEI-CyD and PEI-CyD-tegafur could both condense DNA at the N/P ratio of 3. 1–6 represent the N/P ratios of the lanes, respectively

Formation of nanoparticles facilitates complex diffusion, extravasation through vascular fenestration, and cellular uptake (Mintzer and Simanek, 2009). The particle size of polymer/DNA complex distributed from 100 to 300 nm approximately (Fig. 5a). As the N/P ratio increased from 15 to 35, the mean particle size decreased from 198 to 138 nm while the mean zeta-potential increased from 14.65 to 27.75 mV due to electrostatic interaction (Fig. 5b). The reasonable particle size makes it easy for the complexes to be delivered into the cells and the positive surface charge allows an electrostatic interaction between negatively charged cellular membranes and the positively charged complexes. The complexes were also visualized by TEM and SEM. The TEM image shows that the conjugate condensed plasmid DNA to form compact nanoparticles of a size of 100 to 200 nm at the N/P ratio of 25 (Fig. 5c). The SEM image was used to further visualize the appearance of the complexes and shows that sand-like complexes were uniformly distributed on the basal surface (Fig. 5d).

3.3 In vitro cytotoxicity-induced anti-cancer activity

Cytotoxicities of tegafur and PEI-CyD-tegafur were evaluated via MTT assay. Curves of cell viability as a function of tegafur concentration were fitted using Eq. (2), and the IC_{50} was calculated as shown in Table 1.

Table 1 Parameter (IC_{50}) in fitting curves of MTT assays

Compound	Cell line	IC_{50} (μg/ml)
Tegafur	B16F10, HT29, COS7	–
PEI-CyD-tegafur	B16F10	1.388
PEI-CyD-tegafur	HT29	0.598
PEI-CyD-tegafur	COS7	2.335

The in vitro cytotoxicity tests showed PEI-CyD-tegafur conjugate to be much more toxic than free tegafur to B16F10, HT29, and COS7 cells, an observation consistent with the enhanced anti-cancer activity of PEI-CyD-tegafur. Furthermore, IC_{50} values in B16F10 and HT29 cells (1.388 and 0.598 μg/ml, respectively) were 2–4 fold less than IC_{50} value in COS7 cells, indicating that PEI-CyD-tegafur was more toxic to cancer cells (B16F10 and HT29 cells) than to normal cells (COS7 cells). In case of COS7 cells, it was noteworthy that PEI-CyD-tegafur showed low cytotoxicity as the concentration of tegafur was below 1 μg/ml (Fig. 6).

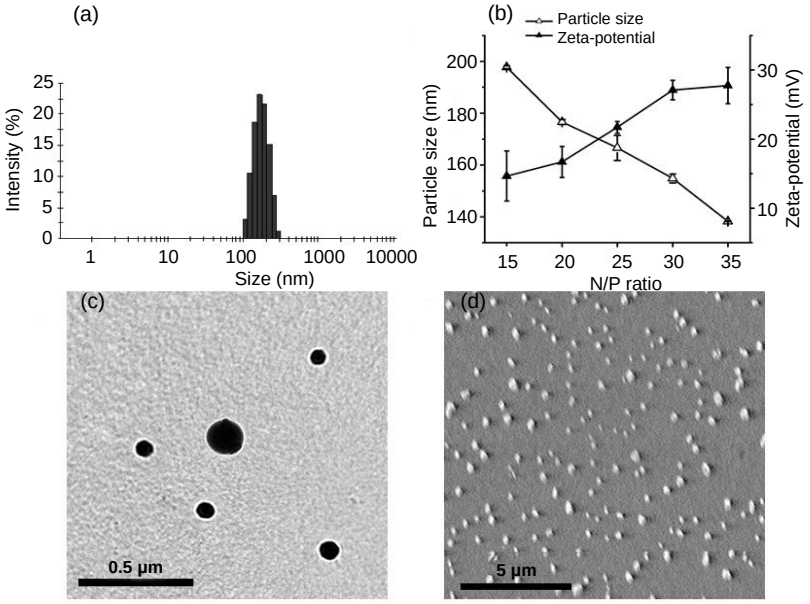


Fig. 5 Physical characterization of PEI-CyD-tegafur/DNA complexes
(a) Size distribution; (b) The mean particle size and the mean zeta-potential at different N/P ratios; (c) TEM image; (d) SEM image

3.4 In vitro transfection of PEI-CyD and PEI-CyD-tegafur conjugates

To investigate in vitro gene transfer capability of CyD-PEI-tegafur, transfection of B16F10 and COS7 cells by pEGFP was carried out. From Fig. 5b we can find that optimized N/P ratios for PEI-CyD and PEI-CyD-tegafur were in the range of 25–30. The N/P ratio of 25 was used in the transfection assays. The fluorescence images of the transfected B16F10 and COS7 cells (Fig. 7) shows that PEI-CyD copolymer had a relatively high gene transfection ability, compared to PEI-25kDa whereas the gene transfection ability of PEI-CyD-tegafur conjugate was lower. When tegafur was conjugated to PEI-CyD, changes of particle size and zeta-potential induced a slight decrease of binding ability and buffer capability as a result of reduced number of primary amino groups on PEI; therefore, the transfection activity of PEI-CyD-tegafur was lower than that of PEI-CyD. However, the potential for gene delivery of PEI-CyD-tegafur was sufficient to conduct a co-delivery of tegafur and plasmid DNA.

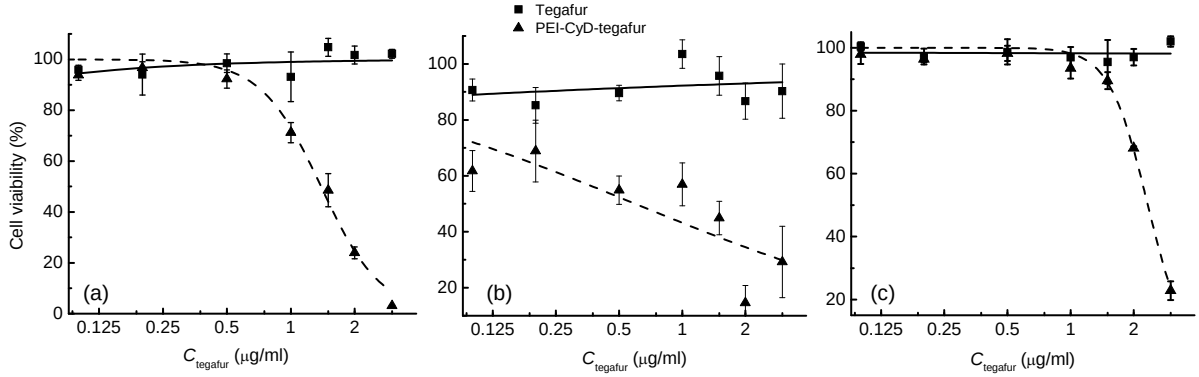


Fig. 6 In vitro cytotoxicities of tegafur and PEI-CyD-tegafur conjugate to B16F10 (a), HT29 (b), and COS7 (c) cell lines

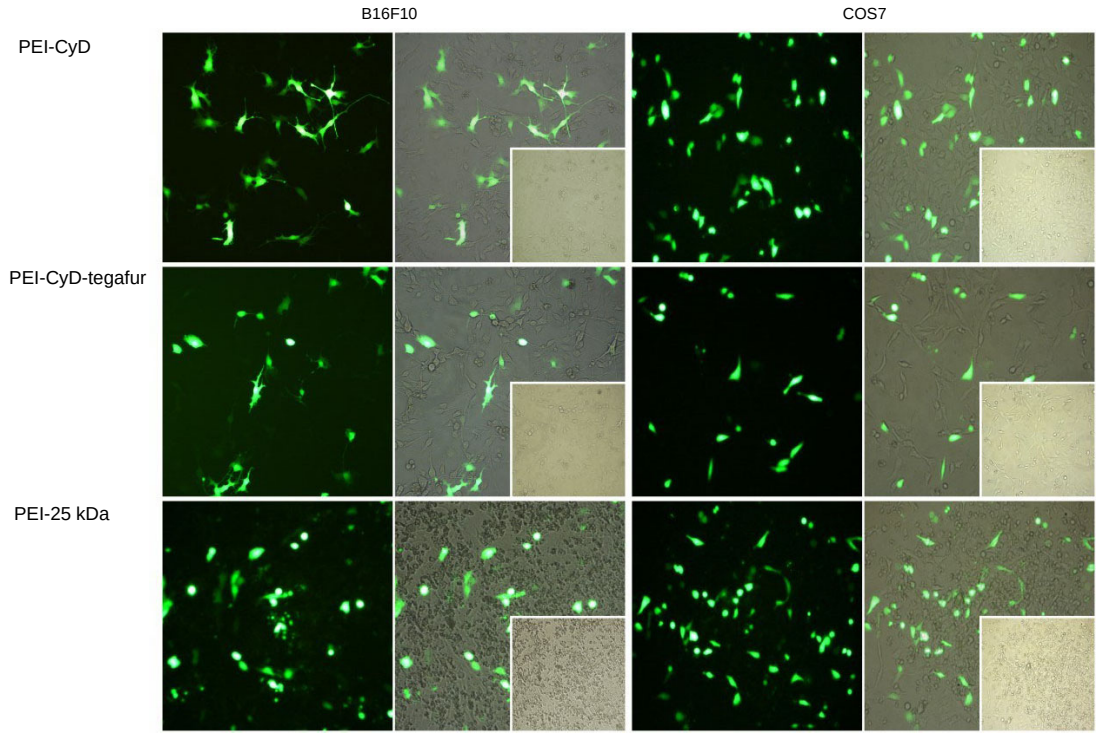


Fig. 7 In vitro transfection of PEI-CyD, PEI-CyD-tegafur, and PEI-25 kDa in B16F10 and COS7 cell lines

4 Discussion

As an ideal delivery vector, PEI-CyD can conjugate with various moieties to form functional materials for gene delivery (Tang *et al.*, 2006), drug delivery (Zhao *et al.*, 2009), and targeted gene transfection (Yao *et al.*, 2009; Huang *et al.*, 2010). Since highly efficient combined therapy calls for co-delivery of anti-cancer chemotherapeutic agents and therapeutic genes, a multi-functional system is needed to simultaneously deliver drugs and genes into cancer cells. Great effort has been made to develop a co-delivery system that has enhanced therapeutic anti-cancer effect. Wang *et al.* (2006) reported the co-delivery of paclitaxel and Bcl-2-targeted siRNA conducted by core/shell nanoparticles. Chen *et al.* (2009) used mesoporous silica nanoparticles to perform co-delivery of doxorubicin and Bcl-2 siRNA which enhances the efficacy of chemotherapy in multidrug-resistant cancer cells. In our study, PEI-CyD-tegafur was synthesized to execute co-delivery of tegafur and plasmid DNA.

In order to conjugate tegafur to PEI-CyD polymer, hydroxyalkylation of tegafur was a necessary step that enabled CDI-induced formation of amido linkage between tegafur and PEI-CyD. Compared to other hydroxyalkylation procedures using toxic chlorohydrine (Shi *et al.*, 2005; Zhang *et al.*, 2007) or bromhydrin (He *et al.*, 2000), hydroxymethylation conducted by formaldehyde takes place in a milder way with high yield. And then hydroxymethyl tegafur activated by CDI was successfully conjugated to PEI-CyD copolymer. Considering the gene delivery ability required, the drug loading amount was purposely reduced to retain considerable primary amine groups for gene delivery. PEI-CyD-tegafur conjugate is a simple and rationally designed delivery system that performs efficient synthesis with only a few steps and assembles with plasmid DNA into sub-300-nm nanoparticles in water, thereby obviating the need for organic solvents during processing.

Biological study shows that the increased cytotoxicity of PEI-CyD-tegafur conjugate induced enhanced anti-cancer activity. Many studies reported that pure PEI-600 has little cytotoxicity as well as low transfection efficiency (Mintzer and Simanek, 2009), and our approach showed PEI-CyD was a low-toxic carrier in vitro (Tang *et al.*, 2006). Therefore, the enhanced cytotoxicity of PEI-CyD-tegafur might be caused by the elevated bioavailability of tegafur, polymer-mediated efficient delivery (Zhao *et al.*, 2009), amine groups in crosslinked PEI (Tang *et al.*, 2006), and auxiliary formaldehyde-induced antineoplastic activity (Engel *et al.*, 2008). Furthermore, the IC_{50} value in cancer cell was 2–4 fold less than that in normal cells, thus conjugates in the same dose could lead to distinctive cytotoxicity in different cell lines, demonstrating that PEI-CyD-tegafur was relatively protective to normal cells and toxic to cancer cells. As a clinical oral chemotherapeutic agent, administration of tegafur leads to extensive body distribution (Malet-Martino and Martino, 2002) which calls for different cytotoxicity between normal cells and cancer cells. PEI-CyD-tegafur conjugate influenced the differences in cytotoxicity to a certain degree. A more efficient method is to conjugate targeting moieties onto PEI-CyD polymers to achieve targeted delivery of anti-cancer agents. Folic acid might be an ideal targeting moiety for drug delivery of PEI-CyD-tegafur. Folate receptor α, primarily expressed in cancerous cells such as malignant nasopharyngeal, colon, ovarian, breast, renal, and testicular carcinomas, can induce targeted drug delivery into the cells via receptor-mediated endocytosis (Yao *et al.*, 2009). Further research on folate conjugate to PEI-CyD-tegafur will be carried out.

For the gene delivery study, the conjugate showed a satisfying result. Endocytosis and lysosomal escape of polymer/DNA complex largely affect the transfection efficiency (Mintzer and Simanek, 2009). The PEI-CyD-tegafur conjugate could be delivered by endocytosis with its positive surface charge and escape from lysosomal enzyme via proton sponge effect supported by primary amine on PEI. Optimized N/P ratio determined by DNA retardation assays was in range of 25–30. Electron microscopy images showed that PEI-CyD-tegafur conjugate was able to condense plasmid DNA into spherical nanoparticles with size of approximately 150 nm which is suitable for delivery system. The gene delivery ability was also supported by in vivo pEGFP transfection assays. PEI-CyD-tegafur showed the potential to deliver gene into cells though the transfection efficiency of the conjugate was slightly lower than PEI-CyD copolymer due to reduction of primary amine.

5 Conclusions

PEI-CyD-tegafur conjugate was synthesized for co-delivery of chemotherapeutic agent tegafur and reporter EGFP plasmid DNA. It allows enhanced cytotoxicity with efficient transfection. With its high cytotoxicity in cancer cells, PEI-CyD-tegafur can significantly improve the anti-cancer activity. The transfection efficiency of PEI-CyD-tegafur makes it possible to deliver therapeutic gene. PEI-CyD-tegafur conjugate fully meets the requirements we have proposed in the introduction section and possesses these six features to be a promising co-delivery system.

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